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Study of Bacterial Resistance in Clinical Isolates of S*taphylococcus aureus* by Comparison of Conventional *in-vitro* Methods with PCR & RFLP Based Genotypic Methods

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Methicillin Resistant *S. aureus* is an increasingly common cause of nosocomial infections, causing severe morbidity and mortality worldwide and accounting for more than 50% of all *S. aureus* diseases. *S. aureus* used in our study were procured isolates from the microbiology lab of a corporate hospital. Reference culture of *S. aureus* NCIM 5021 was obtained from NCIM, Pune. Clinical isolates were subjected to antimicrobial susceptibility testing by standard antibiotic disc (Cefoxitin 30 µg). The selected clinical strains were tested for sensitivity to different antibiotics by Kirby Bauer disc diffusion method. The MIC was determined by two fold serial dilution in broth individually against various antibacterial agents. That was followed by a study on a combination of Ciprofloxacin & Linezolid by two dimensional checkerboard method of synergism testing against the clinical isolates and reference *S. aureus* NCIM culture. FIC index of combination indicated synergism against *MRSA*. *MRSA* was used for PCR based study of the resistant gene namely *mecA*. PCR-RFLP method was used to study 16S rRNA by restriction digestion using *Taq 1*. This is

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indicative of the possible serological differences among clinical isolates and was compared to NCIM culture based on restriction fragments.

Keywords: mecA Gene; FIC Index; PCR; RFLP.

1. INTRODUCTION

Methicillin Resistant *Staphylococcus aureus (MRSA)* is a major nosocomial pathogen causing momentous morbidity and mortality. In India, probably due to overcrowding and poor personal hygiene, CA-MRSA has spread. Although it mainly manifests in severe soft tissue and skin infections requiring surgical drainage, it is now becoming pronounced in bacteremias affecting neonates, especially from lower economic sections, and breast abscesses in lactating mothers, becoming increasingly common in urban areas. In India, the significance of *MRSA* had been recognized reasonably late and it appeared as a problem in the 80s and in the 90s. Recently epidemic strains of these *MRSA* are generally resistant to several other antibiotics [1].

Human-adapted *MRSA* strains can be transmitted to animals in close contact, which can sometimes act as carriers and re-infect people. Especially Methicillin-resistant *S.aureus* strains have acquired the *mecA* gene, which is carried on large mobile genetic element called the *Staphylococcus* chromosomal cassette *mec* (Sccmec). Methicillin was introduced in 1959, and methicillin-resistant *Staphylococcus aureus* (*MRSA*) strains rapidly emerged and became a major problem in hospitals in the 1960s. These nosocomial *MRSA* strains were multidrug resistant (MDR), many being susceptible only to glycopeptides. The past 10 years have seen reports of reduced susceptibility (vancomycin-intermediate *S. aureus* [*VISA*]) and resistance (vancomycin-resistant *S. aureus* [*VRSA*]) to vancomycin. Methicillin-Resistant *S. aureus* (MRSA) strains have acquired a gene that makes them resistant to all beta-lactam antibiotics. *S. aureus* strains have acquired the mecA gene, which is carried on a large mobile genetic element called the *staphylococcus* chromosomal casstte mec (Sccmec). This gene codes for a penicillin binding protein, PBP2a, which interferes with the effects of the beta lactam antibiotics (e.g Penicillins and Cephalosporins) [2].

Combination therapy is used with the aim of expanding the antimicrobial spectrum, minimizing toxicity, preventing the emergence of resistant mutants during therapy and obtaining synergistic antimicrobial activity [3].

2. METHODS

2.1 Bacterial Isolation

50 Strains of *S. aureus* obtained from the Department of Microbiology, Sri Ramakrishna Hospital, Coimbatore, India, were included in the study. Overall, 62% of the isolates were obtained from the respiratory tract, 17% from urine and 21% from a variety of other sources. The isolates were identified by Conventional methods and checked for purity by plating Mannital salt agar. *S. aureus* ATCC 29213 (NCIM-5021) was included as a quality-control strain.

2.2 Antimicrobial Agents

Standard laboratory antibiotic discs of Ciprofloxacin(CIP), Amoxicillin(AMX), Trimethoprim(TR), Clindamycin(CD), Amikacin(AK), Gentamicin(GEN) and Vancomycin(VA) used in this study.

2.3 Screening of the Clinical Isolates of *Staphylococcus aureus* for Methicillin Resistance with Cefoxitin (30 µg) by Disc Diffusion Method

All the clinical isolates of *Staphylococcus aureus* were screened for Methicillin Resistance with Cefoxitin by disc diffusion method according to NCCLS guidelines. The break point of less than ≤ 21 mm for Cefoxitin was indicative of Methicillin resistance. Among all clinical isolates of *Staphylococcus aureus* that were C.I - 1, C.I - 2 and C.I - 3.

2.3.1 Antibiotic susceptibility testing

Antibiotic susceptibility testing was done on Mueller–Hinton (MH) agar using the Kirby–Bauer disc diffusion method with CIP, AMX, TR, CD, AK, GEN, and VA. The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute. The susceptible break points applied were ≤ 20. Isolates of *S.aureus* resistant break point ≥14. [4].

2.3.2 MIC determination

The MICs of each agent alone were determined by broth macrodilution using sterile glass test tubes containing MH broth (supplemented with magnesium and calcium cations). The inoculum contained 56105 c.f.u. ml 21. The concentration ranges tested were: 0.3125 – 40 µg/ml AKml^{-1}, 0.3125-40 µg/ml CIP ml^{-1}. Antimicrobial solutions were prepared and freshly diluted on the day of testing. Each test was performed in duplicate.

2.3.3 Synergy testing

Chequerboard synergy testing was performed in sterile glass test tubes using the macrodilution technique. Antimicrobial solutions were prepared and freshly diluted on the day of the test. Each test was performed in duplicate. Fractional inhibitory concentrations (FICs) were calculated as: (MIC of drug A or B in combination)/ (MIC of drug A or B alone), and the FIC index was obtained by adding the FIC values. FIC values were interpreted as synergistic if values were ≤ 0.5, additive or indifferent for values > 0.5 to 4.0 and antagonistic for values > 4.0. [3,5]

2.4 PCR Amplification for Resistant Genes in Clinical Isolates of Methicillin Resistant

2.4.1 *Staphylococcus aureus*

The mecA gene was amplified using the primers as described by [6]. PCR products were analyzed in 1.2% agarose gel and premixed with ethidium bromide suspension (10 µl). The PCR products were observed under UV transilluminator after electrophoresis.

Primers	Sequence	Length
Forward	5'-GTA GAA ATG ACT GAACGT CC-3'	20 bases
Reverse	5'-CCA ATT CCA CAT TGTTTC GG-3'	20 bases

Composition of PCR reaction mix

S. no	Composition	Quantity
1	Distilled water	40 µl
2	Assay buffer	5 µl
3	dNTPs	2 µl
4	Forward primer	0.1 µl
5	Reverse primer	0.1 µl
6	Template DNA	2 µl
7	*Taq* polymerase	1 µl
8	Total	50.2 µl

PCR conditions for 30 cycles

S. no		Temperature	Time
1	Initial Denaturation	94℃	2 minutes
2	Denaturation	94℃	30 seconds
3	Annealing	52℃	30 seconds
4	Extension	72℃	1 minute
5	Final extension	72℃	5 minutes

2.4.2 PCR-RFLP method

The 16s RNA was amplified using the primers as provided by [7]. PCR mixture consists of 0.1 µl of each primer (5'-AGAGTTGATCCTGGCTCAG-3') and (5'-ACGGCTACCTTGTTACGAC'-3). PCR products were analyzed in 1.2% agarose gel and premixed with ethidium bromide suspension (10 µl). The PCR products were observed under UV transilluminator after electrophoresis.

Primer for 16S rRNA amplification

Primers	Sequence	Length
Forward	5'-AGAGTTGATCCTGGCTCAG'-3	19 bases
Reverse	5'- ACGGCTACCTTGTTACGAC'-3	19 bases

Composition of PCR reaction mix

S. No	Composition	Quantity
1	Distilled water	40 µl
2	Assay buffer	5 µl
3	dNTPs	2 µl
4	Forward primer	0.1 µl
5	Reverse primer	0.1 µl
6	Template	2 µl
7	*Taq* polymerase	1 µl
8	Total	50.2 µl

PCR conditions for 30 cycles

	Temperature	Time
Initial denaturation	94℃	2 minutes
Denaturation	94℃	30 seconds
Annealing	58℃	30 seconds
Extension	72℃	90 seconds
Final extension	72℃	5 minutes

2.5 Restriction Digestion

Restriction digestion of 16s rRNA was carried out with *Taq* 1(*Thermus aquaticus*) restriction enzyme. Reaction mixture consisted 20 µl of Distilled water, 4 µl of Assay buffer, 15 µl of DNA and 2 µl *Taq* 1 restriction enzyme. Restriction digestion was carried out for 40 µl of reaction mixture at 65°C for 2 hr. Restriction digestion fragments were observed under UV transilluminator after gel electrophoresis.

S. No	Composition	Quantity
1	Distilled water	20 µl
2	Assay buffer	4 µl
3	DNA	15 µl
4	*Taq* 1 enzyme	2 µl

3. RESULTS

PCR technique was applied to three clinical isolates and one NCIM culture (C.I-1, C.I-2, C.I-3 and NCIM). The *mecA* gene DNA fragment of 500bp was targeted and amplified from the isolated DNA by using thermo cycler (PCR). *mecA* gene was observed in only C.I - 3 and that was confirmed by Agarose gel electrophoresis.

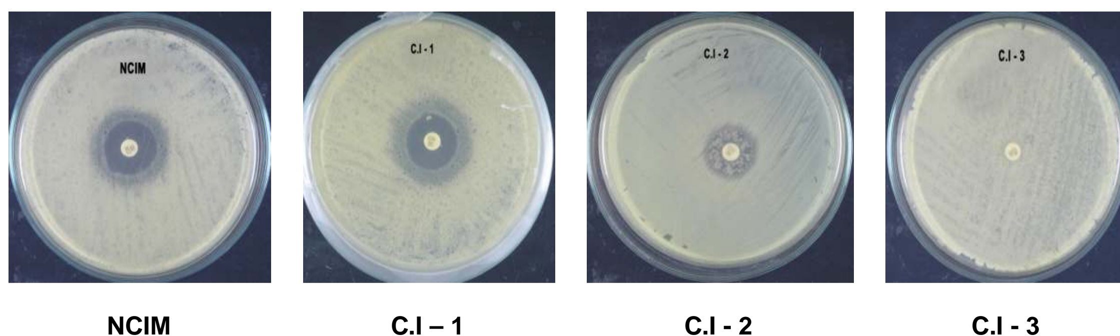
Table 1. Screening of the clinical isolates of *Staphylococcus aureus* for methicillin resistance with cefoxitin (30 µg) by disc diffusion method

S. no	Standard antibacterial disc concentration (µg)	Zone of inhibition in mm (n=3)			
		NCIM	C.I – 1	C.I – 2	C.I – 3
1	Cefoxitin 30 µg	22 mm	R	R	R

NCIM= National collection industrial microorganisms, C.I = Clinical isolate, R= Resistance, all Clinical isolates tested n=3

Table 2. Interpretive criteria (in mm) for cefoxitin disk diffusion test [8]

S. no		Susceptible	Resistant
1	*S. aureus*	≥ 22 mm	≤ 21 mm
2	Coagulase negative *S. aureus*	≥ 25 mm	≤ 24 mm

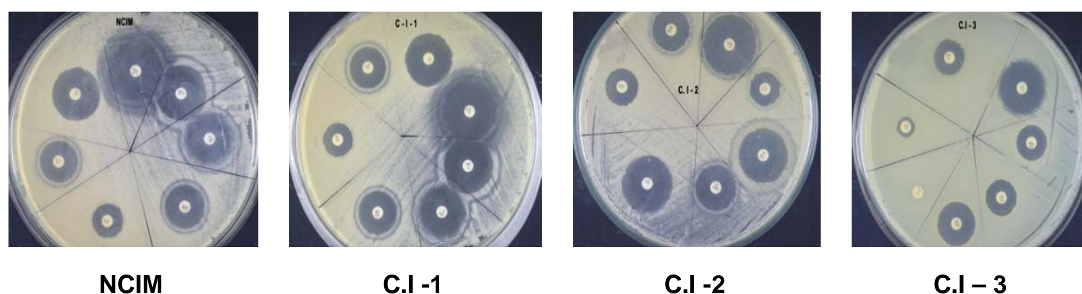


NCIM **C.I – 1** **C.I - 2** **C.I - 3**

Fig. 1. Screening of the clinical isolates of *Staphylococcus aureus* for methicillin resistant with cefoxitin 30 µg by disc diffusion method

Table 3. Susceptibility testing of the clinical isolates of *MRSA* by Kirby Bauer method

S. no	Standard antibacterial discs	Zone of inhibition in mm (n=3)			
		NCIM	C.I – 1	C.I – 2	C.I – 3
1	Amoxicillin 10 µg	34	19	16	10
2	Ciprofloxacin 5 µg	28	18	20	18
3	Trimethoprim 5 µg	27	20	20	R
4	Clindamycin 2 µg	27	20	17	20
5	Amikacin 30 µg	22	19	16	12
6	Gentamicin 10 µg	22	21	18	17
7	Vancomycin 30 µg	24	17	19	18



NCIM **C.I -1** **C.I -2** **C.I – 3**

Fig. 2. Susceptibility pattern of *MRSA* to various antibiotics by Kirby Bauer method

Table 4. Determination of Minimum Inhibitory Concentration (MIC) of antibacterial agents against clinical isolates of *MRSA* by macro dilution method (n=2)

S.no	Drug & MIC values	C.I-1	C.I-2	C.I-3	NCIM
1	Amikacin µg/ml	5	10	20	1.25
2	Ciprofloxacin µg/ml	2.5	5	40	1.25
3	Linezolid µg/ml	2.5	5	20	0.625
4	Conc. range used	0.3125-40	0.3125-40	0.3125-40	0.3125-40

Table 5. Synergy testing of combination involving two antibacterial agents by Checkerboard method (n=2), (A=Ciprofloxacin), (B=Linezolid) [9]

S. no	*S. aureus* strains	CIP+LNZ (FIC index)
1	C.I – 1	0.5
2	C.I – 2	0.4
3	C.I – 3	0.5

No antagonistic FIC indices > 4 were found. FIC indices ≤ 0.5 were considered Synergistic, whilst those >0.5–4 were considered additive or indifferent

4. DISCUSSION

Resistance to methicillin is determined by the function of penicillin-binding protein 2’ (PBP2’, or PBP2a) that binds to β-lactam antibiotics with much lower affinity than the intrinsic set of PBPs of *S. aureus.* PBP2’ is encoded by the methicillin resistance gene *mec*A located in the chromosome of *MRSA* on mobile genetic element designated staphylococcal cassette chromosome *mec* (SCC*mec*). Besides *mec*A gene, SCC*mec* element often contains genes responsible for resistance to antibiotics other than β-lactams. Currently, eleven SCC*mec* types have been described by the “International Working Group on the Classification of Staphylococcal Cassette Chromosome (SCC) Elements (IWG-SCC). SCC*mec* types I, IV and V encode exclusively for resistance to β-lactam antibiotics, while SCC*mec* types II and III determine multidrug-resistance owing to the presence of additional drug resistance genes on integrated plasmids pUB110 (resistance to kanamycin, tobramycin and bleomycin) and pT181 (resistance to tetracyclin) or transposon Tn554 (inducible MLS resistance). Resistance to antibiotics other than β-lactams in strains SCC*mec* type.

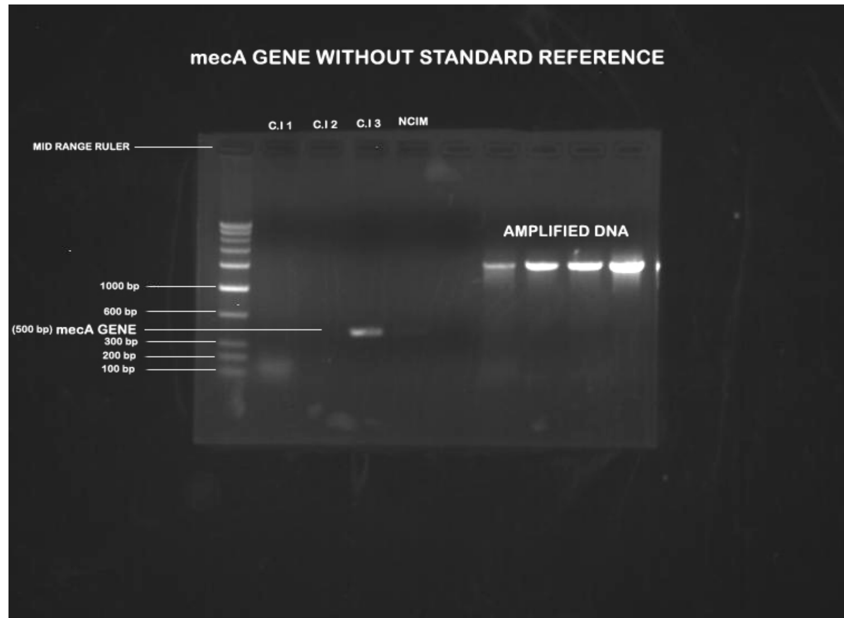


Fig. 3. *mecA* gene detection in the selected strains of MRSA

(From left to right)
Lane 1: Mid range ruler (100 bp to 1000 bp)
Lane 2: C.I- 1
Lane 3: C.I- 2
Lane 4: C.I- 3
Lane 5: NCIM
Lane 7 to 10: Amplified DNA of the above strains

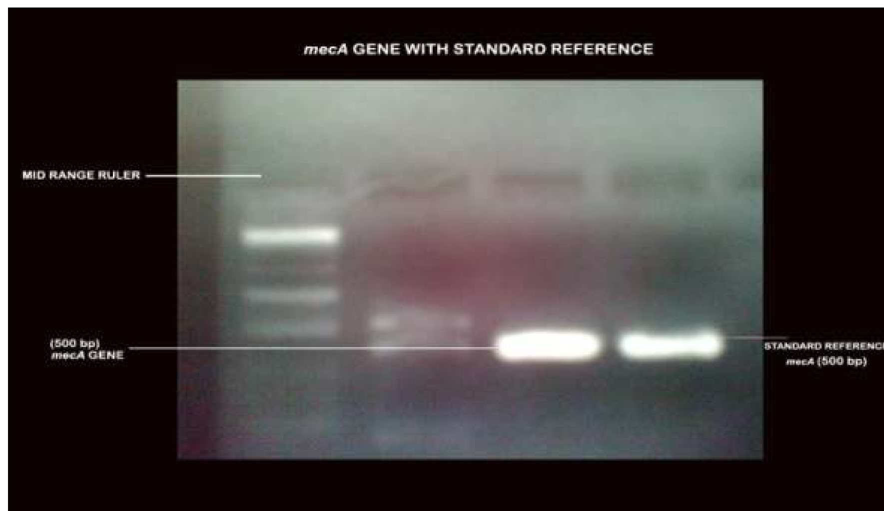


Fig. 4. Comparison of MRSA gene of clinical isolates with reference strain by agarose gel electrophoresis

(From left to right)
Lane 1: Mid range ruler (100 bp to 1000 bp)
Lane 2: Negative Control
Lane 3: MRSA C.I-3
Lane 4: MRSA reference strain for mecA

Fig. 5. Restriction fragments similarity of 16S rRNA Gene of different *Staphylococcus aureus* followed by PCR-RFLP method

Table 6. Screening and confirmation of methicillin resistance in clinical isolates of *Staphylococcus aureus*

S. No	Standard antibacterial disc	Zone of inhibition in mm (n=3)			
		NCIM	C.I – 1	C.I – 2	C.I – 3
1	Cefoxitin 30 µg	22	1	R	R
2	Identification of mecA gene by PCR	Absence of mecA	Absence of mecA	Absence of mecA	Presence of mecA

Synergism of chalcones and non- β-lactam antibiotics cannot be easily explained. Other mechanisms could be inhibition of β-lactamase, inactivation of efflux pumps, destabilization of cytoplasmic membrane, disruption of PBP2' synthesis and inhibition of topoisomerase.

Synergy testing methods are not standardized for reproducibility and interpretation, and therefore it is extremely difficult to compare the results of these methods from different studies. The checkerboard test measures only the inhibitory concentration. The time parameter of 24 h can limit or alter the results of an experiment if regrowth occurs with one or both antimicrobial agents.

Result of study involving Ciprofloxacin in combination with Linezolide respectively (by checkerboard method) was found synergistic against clinical isolates of *S. aureus*. Conclusion of our study exhibited synergy in the combinations CIP+LNZ against clinical isolates of *S. aureus*. Although it is interesting that we could demonstrate *in- vitro* synergy against some *S. aureus* isolates, the mechanism of the exhibited synergy is unknown and needs to be explored. Also, no evidence of *in vivo* synergy has been found. Clinical studies are necessary to test the validity of these in vitro findings, as well as the significance of regrowth after 24 h.

The result of screening of MRSA isolates by Cefoxitin disc diffusion method as per CDC was further justified for the presence of resistance by the identification of mecA gene that was confirmed by PCR based method. Thus Cefoxitin disc diffusion method can be considered as a suitable and simple method for initial detection of MRSA.

5. CONCLUSION

Comparative 16S rRNA digestive pattern of the Clinical isolates of *MRSA* and reference strain, *Staphylococcus aureus* NCIM-5021 were studied by PCR-RFLP method. In this, two different restriction fragments were obtained as can be inferred by the difference in the agarose gel electrophoresis separation pattern. A PCR-RFLP variation occurs if PCR amplicons or the fragments that result after restriction digest of such amplicons have a different length distribution among the input genomes. Fragment length distributions differ if they have different numbers of fragments or if any of the fragments differ in length. This indicates Clinical *Staphylococcus aureus* (lane2 and 3) and NCIM

Culture (lane 5) are serologically different[5]. Linezolid plus Ciprofloxacin seemed to be the most active combination against MRSA strains in Checkerboard broth dilution method. Good activity of Linezolid and Ciprofloxacin on the *MRSA* strain suggests that this combination would be a reasonable therapeutic choice for patients. Molecular techniques are the most sensitive method in detecting *S. aureus at both genus* and species level and with 100% accuracy in detecting MRSA, when compared with the classical identification method. *In-vivo* studies are needed to validate the *in-vitro* observation.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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